

Lactation induction as a predictor of post-parturition transgene expression in bovine milk

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The bovine's long generation interval results in a delay of several years when evaluating mammary specific transgenes in genetically engineered animals. This experiment was conducted to evaluate the feasibility of reducing that waiting period. Lactation was induced in prepubertal bull and heifer calves as a means of predicting transgene behaviour during subsequent post-parturient lactations in the heifers themselves, and in daughters sired by the bulls. The animals carry a lactation-specific transgene encoding lysostaphin, an antimicrobial protein that kills *Staphylococcus aureus*, a mastitis-causing pathogen. Oestrogen, progesterone and dexamethasone were administered as previously described (Ball et al. 2000) to nine heifers (five transgenics) ranging in weight from 80 to 145 kg. Eight bull calves (seven transgenics) weighing 81–178 kg received additional oestrogen and progesterone injection prior to dexamethasone treatment. All nine heifers responded to the milk induction scheme yielding between 19 ml and 4.5 l over 5 d. Milk volume from the four responding males (30 µl to 2.5 ml) was significantly less than that harvested from females ($P=0.025$). Only bull calves >117 kg had a positive response. Lysostaphin was detected in all transgenic prepubertal heifers and in two transgenic prepubertal bull calves induced. A positive relationship was observed between lysostaphin's stapholytic activity in the two types of lactations ($r^2=0.907$, $P<0.001$) thus providing a useful means of predicting subsequent lysostaphin production in *post-partum* milk.

Keywords: Bull, secretion, mastitis, prepubertal.

Genetically engineered livestock's promise to bring new food and biomedical products to the marketplace is hampered by the lengthy generation interval of most species. Not only does this slow product development, but it also causes significant initial product production delays. The time commitment for transgenic cattle projects is particularly burdensome, especially for those projects involving harvest of transgene products from mammary gland secretions. In such mammary gland projects, the time from transgenic embryo production to evaluation of milk is at least twice the gestation length of the target species plus the time from birth to puberty (cattle, 31 months; sheep and goats, 17 months; swine, 15 months). Once a useful founder animal has been identified, time equivalent to another generation interval must be invested to propagate a production herd. The time required to achieve these milestones, using standard breeding techniques, can exceed 5 years for cattle.

Radical strategies have been proposed to shorten significantly the time interval from project initiation to product harvest. They include expressing the transgene in the urinary bladder (Kerr et al. 1998) or in blood (Logan & Martin, 1994), so that product can be harvested from both males and females at any time after birth. Those and other alternatives have not been competitive with mammary gland bioreactors either because of low product yield, cumbersome harvest procedures, or product purification difficulties. A number of more conventional reproductive strategies have been proposed to reduce the time required to identify, evaluate and expand successful lines of transgenic dairy animals (Bondioli & Wall, 1998). Assisted reproductive techniques (ART) can be used to shorten the generation interval by months. But those approaches only marginally decrease the time investment required to evaluate transgene product levels. Utilizing a strategy that can identify founders capable of producing productive offspring in a timely manner not only serves as an adjunct to ART for decreasing time to production, but it also allows resources to be focused on the most important animals in

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an earlier phase of the project. An encouraging study has demonstrated the possibility of measuring transgene expression in secretions (witch's milk) from newborn transgenic rats (Hirabayashi et al. 1996). More traditionally, researchers have induced lactation in prepubertal transgenic does (Ebert et al. 1994; Baguisi et al. 1999; Cammuso et al. 2000; Baldassarre et al. 2004) and pigs (Shamay et al. 1992). In both species, milk from induced lactations was at least qualitatively predictive of levels of transgene expression in post-parturition milk samples. Recently, transgene expression has been evaluated in milk from transgenic cattle induced to lactate (Van Berkel et al. 2002; Brophy et al. 2003). The goal of those lactation induction studies was to establish a means of predicting transgene expression levels during natural lactation. The study reported here extends those findings by reporting on transgene expression in secretions following induced lactations and post-parturition lactations in the same animals. Furthermore, an attempt was made to demonstrate the feasibility of predicting transgene expression levels of daughters of male founders by evaluating induced mammary gland secretions in the prepubertal transgenic founder bulls. If induced mammary gland secretions from prepubertal bulls accurately predict daughter's *post-partum* transgene performance, it would be possible to make a decision on the merits of propagating a line without first going to the expense of breeding and raising animals to test. Additionally, the decision could be made several years earlier. Of course the induction protocol would have to be shown to be harmless to future reproductive performance of the bulls. Finally, as a way of determining whether induced lactations reflect normal milk composition, endogenous milk constituents were compared in induced secretions and post-parturition milk.

Materials and Methods

Production of transgenic cattle

The transgenic Jersey cattle included in this study were produced as previously described (Powell et al. 2004). They carry a transgene construct that includes genes for neomycin resistance, green fluorescent protein and a peptidoglycan hydrolase (lysostaphin) with expression directed to mammary gland secretory epithelium. Expression of the modified lysostaphin gene (Kerr et al. 2001) is regulated by an ovine β -lactoglobulin promoter (a gift from AJ Clark, pBJ41, Roslin Institute, UK). Nine heifers and eight bull calves were used in this study. The three transgenic founder heifers (001, 101, and 204) evaluated in this study were all cloned from the same Jersey fetus but each was produced on different days from newly transfected donor fibroblasts. Their transgene expression has been characterized (Wall et al. 2005). Semen from two transgenic founders was used to produce two second generation heifers (215 and 312). Six transgenic bulls calves were sired by one founder bull and

one bull calf each was produced by two other founder bulls.

All three founder bulls who generated offspring for this study were cloned from the same Jersey fetus but from independently transfected donor fibroblasts; thus, all founder animals' transgene locus differ in both location and copy number. This strategy was intended to produce animals with different levels of transgenic expression. Four unrelated age-matched non-transgenic, non-clone Jersey heifers and one non-transgenic Jersey bull calf served as controls. The production, propagation and experimentation involving live animals was approved in advance by the Beltsville Animal Care and Use Committee.

Lactation induction

Prepubertal heifers, 5–8 months of age and weighing 100–150 kg were induced to lactate as previously described (Ball et al. 2000). Bulls 3–7 months of age, weighing 80–190 kg, were induced to lactate using a slightly modified induction protocol (Suyapa Ball, personal communication). Briefly, bulls were given twice daily subcutaneous injections of oestradiol-17 β (0.1 mg/kg body weight, Cat. No. 437, Steraloids, Newport RI, USA) and progesterone (0.25 mg/kg, Cat No. p-0130, Sigma, St. Louis MO, USA) for 14 d followed by intramuscular injections of reserpine (2.5 mg/d, Prescription Specialties, Knoxville TN, USA) on days 15–17 inclusive. Reserpine was used to stimulate prolactin secretion and thus enhance milk production (Collier et al. 1976). Dexamethasone (20 mg/d, Butler, New Platz NY, USA) was administered intramuscularly on days 18–20 inclusive.

Milk sample collection

An attempt was made to collect induced lactation secretions aseptically by hand milking twice daily for 5 d, following the last hormone injection. Not all animals yielded secretions at all ten milkings. Samples were stored at -20°C and were centrifuged at 800 *g* for 15 min after thawing and before being analysed. Post-parturient samples were collected with the aid of an automated sampling device during machine milking. All natural lactation samples, collected on or about days 30, 60 and 90 *post partum* at morning milkings, were centrifuged at 800 *g* at 4°C for 15 min and the infranatant (skim milk) was tested immediately or frozen at -20°C until assayed.

Lysostaphin biological activity assay

Spot-On-Lawn (SOL) assays were performed to compare the stapholytic activity of milk from transgenic cows with that of recombinant lysostaphin (Sigma, CAS Number: 9011-93-2) as previously described (Wall et al. 2005). Briefly, approximately 1 million colony-forming units of *Staphylococcus aureus* bacteria were overlaid on gridded agar plates and allowed to dry for 30 min at room

temperature before 10- μ l aliquots of diluted test samples or standards were pipetted onto the developing lawn of bacteria. After overnight incubation at 37 °C, optical density of cleared zones was measured and interpreted using a four parameter logistic standard curve (Hill plot, Prism 4, GraphPad Software, Inc., San Diego CA, USA). Samples were assayed in triplicate and a dilution series of recombinant lysostaphin was always included on each assay plate. The activity of the lysostaphin variant produced by transgenic animals is approximately 20% that of the native bacterial form (Kerr et al. 2001).

Enzyme-linked immunosorbent assays (ELISA)

The skim milk (infranatant) fraction was analysed. Samples were assayed in duplicate using reagents and protocols supplied by the manufacturer (Bethyl, Montgomery TX, USA, E101). Absorbance from the horseradish peroxidase-antibody conjugate-based assays was read at 450 nm with a Spectra Max 340 (Molecular Devices). Standard curves were fitted to a four parameter logistic model (SoftMax Pro, Molecular Devices, Sunnyvale CA, USA). Sample and second antibodies dilutions are as follows: bovine α -lactalbumin (α -la), sample dilution 1:200 000, antibody diluted 1:50 000, (Bethyl Laboratories, E10-128); bovine β -lactoglobulin (β -lg), sample dilution 1:200 000, antibody dilution 1:100 000, (Bethyl Laboratories, E10-125); bovine lactoferrin (LF), sample dilution 1:5 000, antibody dilution 1:100 000 (Bethyl Laboratories, E10-126); lysostaphin, sample dilutions 1:500 and 1:1000, rabbit anti-lysostaphin antibody dilution 1:20 000 as previously reported (Kerr et al. 2001). Results of the lysostaphin ELISA and that of the SOL assay are directly comparable since the same recombinant lysostaphin (Sigma, CAS Number: 9011-93-2) was used to generate standard curves for both assays.

Protein assays

The general pattern of milk proteins (50 μ g/lane) was visualized after 15% SDS-PAGE separation and Coomassie blue staining. Total protein in milk samples was assayed using the bicinchoninic acid (BCA) method (Pierce, Rockford IL, USA) with bovine serum albumin (BSA) as standard.

Lactose/D-galactose assay

Skim milk samples were deproteinized and diluted 1:1000 before analysis. The β -galactosidase-based assay measured conversion of NAD to NADH (Boehringer Mannheim, Indianapolis, USA, Cat. No. 10 176 303 035) and was performed in a 96-well microtitre plate format with a total reaction volume of 0.25 ml. A dilution series of lactose was used to generate a standard curve. Absorbance was read with a model HTS 7000 Bio Plate Reader (Perkin Elmer, Wellesley MA, USA) at 340 nm.

Sperm motion analysis

To determine whether the lactation induction scheme administered to prepubertal bull calves had an impact on semen quality, semen (four ejaculates) was collected from two half brothers 214 and 303 (same transgenic father, 004) one successfully induced to lactation and one not treated. Sperm motion parameters were determined using a Hobson Sperm Tracker for boar sperm analysis (Holt et al. 1996) and *in-vitro* blastocyst production was compared by previously described protocols (Hawk & Wall, 1994; Powell et al. 2004).

Statistical analysis

General Linear Model of Analysis of Variance was performed on quantitative data to identify main effect and first order interaction differences (SPSS 13 for Windows, SPSS Inc., Chicago IL, USA). Effects such as sex, age, genotype and lactation type were included in the model as appropriate. An initial analysis indicated that there were no significant differences in milk component values for samples collected at days 1–5 inclusive following lactation induction or at 30, 60 and 90 d *post partum*. Therefore, for lactation type comparisons, samples collected at 30 d were compared with pooled induced lactation samples values, which were thought to serve as the best representation of the induced secretions. Normalizing milk constituents against total protein did not alter the outcome of the analysis. Values and analysis results are presented without normalization. Fisher's exact test was used to analyse induction success rate proportions. Pearson correlations were computed for the relationship between ELISA and SOL. Linear regression analysis was used to evaluate the relationships between lysostaphin in induced secretions and *post-partum* milk samples (Sigma Plot 2004 for Windows, v.9.0, Systat Software, Inc., Richmond CA, USA). Least square means and their *se* are presented unless otherwise stated. Type I error (α level) was set at 0.05.

Results

Effectiveness of lactation induction

Twelve transgenic and five non-transgenic calves (nine females and eight males) 3–8 months old were hormonally treated with oestradiol, progesterone and dexamethasone to stimulate mammary gland development and lactogenesis. Four of eight males and nine of nine females yielded mammary gland secretions for an overall positive response rate of 76%. All of the females were successfully induced but one (312) did not respond when first induced at 4.5 months (98 kg). She did respond to a second series of injections administered at 5.5 months (128 kg). The number of animals treated was small, but adequate to demonstrate an influence of sex on successful lactation induction ($P=0.0498$). Males that did produce mammary gland

Table 1. Five-day yield (ml) from transgenic and non-transgenic heifers and bull calves following a lactation induction regime

Transgenic calves				Non-transgenic calves			
Females	Yield	Males	Yield	Females§	Yield	Males	Yield
001 (001)†	283.0	004 (004)	0	003 (wildtype)	1480.0	418 (210)¶	1.15
101 (101)	179.0	214 (004)	2.5	102 (wildtype)	704.0		
204 (204)	810.0	216 (004)	0.03	207 (wildtype)	4500.0		
215 (004)	238.5	217 (004)	0	208 (wildtype)	1430.0		
312 (104)‡	18.5	304 (004)	0				
		305 (004)	0				
		419 (210)	0.10				

†ID of experimental animal (ID of the transgenic founder parent)

‡This heifer did not respond to the initial series of injections at 4.5 months of age (134 d) but was successfully induced to lactate after a second round of injections administered at 5.5 months of age (162 d)

§Both parents were non-clones and non-transgenic Jerseys

¶Sire 210 was a transgenic clone, but this offspring was neither transgenic or a clone

secretions were >4 months old and weighed at least 117 kg. Younger and lighter bull calves were not responsive. Heifer calves yielded more milk than bull calves over the 5-d collection period (1185 ± 487 ml v. 1.3 ± 0.7 ml, $P=0.025$, Table 1). Overall, there was no evidence that carrying the lysostaphin transgene influenced the success of induction or amount of milk produced during the induced lactation ($P=0.077$). However, when only females are considered, less milk was recovered from transgenic heifers ($P=0.033$), primarily as the result of an exceptional yield (4.5 l) from one non-transgenic heifer.

Even though variation in volume from animal to animal was substantial and animal numbers were small, differences in the amount of milk collected during induced lactations from non-transgenic (2211 ± 1163 ml) and transgenic (312 ± 134 ml) heifers were detected ($P=0.033$).

Lysostaphin concentration in milk from induced and post-parturition lactations

To determine whether concentrations of a transgene product, lysostaphin, in induced lactation samples are predictive of transgene product concentrations in post-parturition milk, lysostaphin was measured by two independent methods: an ELISA, which provides an estimate of the mass of lysostaphin in samples, and a SOL assay, which measures stapholytic activity of the samples.

Lysostaphin concentration, in both induced and post-parturition samples, tended to be elevated in the first day's secretions (9.7 ± 2.7 µg/ml) compared with the fifth day's secretions (2.5 ± 8.7 µg/ml) but large variations in concentration from day to day precluded detecting a significant day effect ($P=0.599$) as measured by ELISA. A similar, non-significant trend was measured by the SOL assay. Therefore, collection day of induced secretions was dropped from the statistical model comparisons between induced samples and post-parturition milk.

Analysis of the ELISA results revealed a cow \times lactation type interaction (Table 2, $P=0.001$). The highest expressing cows had a higher concentration of lysostaphin

Table 2. Lysostaphin concentration as measured by ELISA and its biological activity as measured by Spot-On-Lawn assay in milk of transgenic heifers collected during induced and natural lactations (Means \pm SEM µg/ml)

Cow	ELISA†		Spot-On-Lawn‡	
	Induced	Natural	Induced	Natural
001	1.4 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	0.1 ± 0.2
101	15.2 ± 0.9	11.0 ± 0.7	2.0 ± 0.2	2.6 ± 0.2
204	0.9 ± 0.7	0.7 ± 0.7	0.1 ± 0.1	0.1 ± 0.2
215	6.2 ± 0.9	1.9 ± 0.7	0.6 ± 0.1	0.8 ± 0.2
312	10.5 ± 0.9	3.6 ± 0.6	0.6 ± 0.2	2.4 ± 0.1

†Cow \times lactation type interaction as measured by ELISA ($P=0.001$)

‡Cow \times lactation type interaction as measured by SOL ($P<0.001$)

in secretions collected following induced lactations than in milk from post-parturition lactations, whereas no difference was detected in lysostaphin concentrations in induced and post-parturition samples in the lowest expressing cow ($P=0.481$).

The SOL assay also detected a cow \times lactation type interaction ($P<0.001$, Table 2). However, the relationship was slightly different than that described by ELISA. Based on the SOL assay the top two expressing cows (101 and 312) had higher stapholytic activity in post-parturition milk samples than in secretions from the induced lactations. For the three lower expressors (001, 204 and 215) the stapholytic activity in induced and post-parturition samples did not differ ($P>0.444$, Table 2).

Although it is recognized that the two assays measure different properties of lysostaphin, the correlation between the two assays was strong for milk samples collected *post partum* ($r^2=0.962$, $P<0.001$). However, the correlation between the two assay methods was not strong for the samples collected following induction ($r^2=0.692$, $P=0.009$, and see Table 2) and may be related to interference caused by the much higher total protein content or opacity in the induced lactation samples, or to other matrix effects.

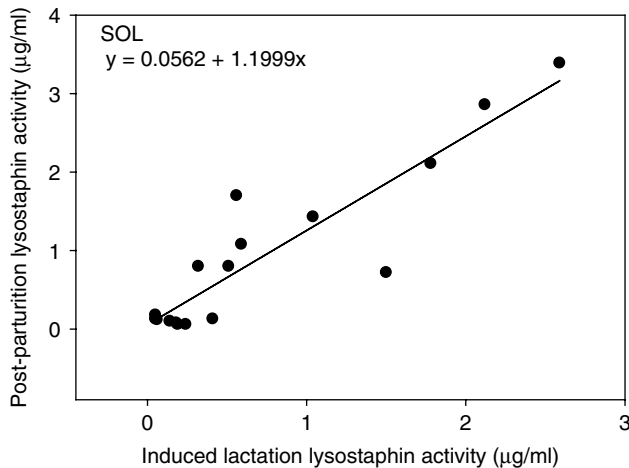


Fig. 1. Lysostaphin concentration and lytic activity in hormonally induced and post-parturition lactations. Lysostaphin stapholytic activity measured by Spot-on-lawn assay ($r^2=0.907$, $P<0.001$).

Transgene product in induced lactation samples as predictor of transgene expression in post-parturition milk

Lysostaphin concentrations in induced secretions, as measured by ELISA, were positively correlated with lysostaphin concentration in post-parturition milk samples, but only weakly so (results not shown). On the other hand, lytic activity in induced samples had a moderately strong linear association with the stapholytic activity in post-parturition milk and therefore might prove to be a good prediction tool ($r^2=0.907$, Fig. 1).

Secretions were collected from four of the eight bulls subjected to the lactation induction protocol. However, only one of the three responding transgenic bull calves produced sufficient volume of secretion to measure lysostaphin concentration. His two samples contained an average of 5.2 ± 0.5 µg/ml lysostaphin as measured by ELISA. His half sibling sister 215 (same transgenic founder sire) had 6.2 ± 0.9 µg/ml lysostaphin in her induced lactation samples.

Milk constituents in induced and post-parturition samples

Milk proteins from induced and natural lactations, separated on polyacrylamide and stained with Coomassie blue, displayed several obvious differences (Fig. 2). Caseins were identified and quantified by densitometry of Coomassie stained PAGE. Separation of α_{s1} -CN and α_{s2} -CN was incomplete. A ratio approximating 4:4:1 for α_s -CN: β -CN: κ -CN was observed in the induced secretions and casein ratio of 2:2:1 was observed in postpartum mid-lactation samples (Fig. 2).

Bands at approximately 18 and 60 kDa appear to be more pronounced in the samples collected following induced lactations. Relative migration of those bands suggests they may represent β -lg and serum albumin

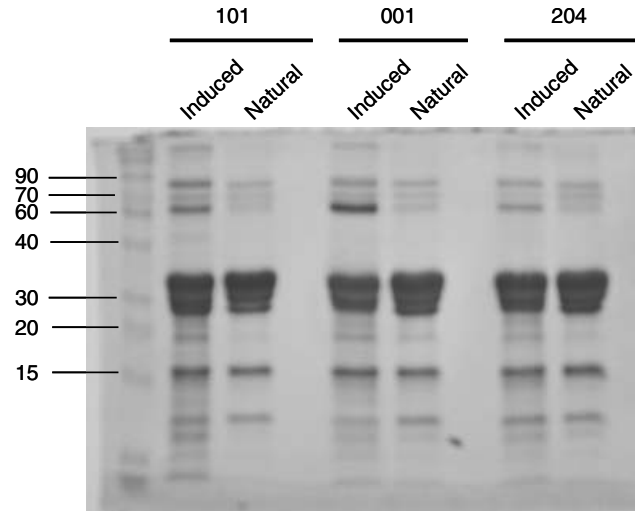


Fig. 2. Protein analysis of milk collected following lactation induction and the first post-parturition lactation from transgenic Jersey cows 001, 101 and 204. Fifty micrograms of total proteins from milk samples were separated on a 15% polyacrylamide gel and visualized by Coomassie blue staining. Lane 1, molecular weight markers (kDa).

respectively. ELISA confirmed that β -lg and serum albumin were more concentrated in post-induction secretions than in post-parturition milk (Table 3). Concentrations of the major whey proteins as well as lactose and an estimate of total protein concentration are presented in Table 3. α -La concentration did not differ in samples from induced and natural lactations. However, β -lg, LF and total protein were more concentrated in the milk from induced lactation than in milk from post-parturition lactations. Conversely, lactose, the primary carbohydrate in bovine milk, was less concentrated in the induced lactation milk than in post-parturition milk.

Genotype of the animals did not have a detectable influence on concentrations of milk proteins measured. However, lactose concentration was higher in the non-transgenic control animals (Table 3).

Serum albumin did not follow the same pattern as the other proteins (Table 3). Samples from induced lactations of transgenic animals had higher (1.87 ± 0.35 mg/ml) serum albumin concentrations than their non-transgenic counterparts (0.18 ± 0.40 mg/ml), whereas transgenic animal post-parturition milk samples had essentially the same (0.17 ± 0.27 mg/ml) concentration of albumin as did the milk samples from non-transgenic cows (0.20 ± 0.30 mg/ml, $P=0.011$ for the genotype \times lactation type interaction).

Customarily milk constituent values are normalized before treatment effects are compared. When this data set of endogenous milk components was normalized, using total protein as the denominator, arcsine transformed and analysed, the statistical inferences as reported above were unchanged. Furthermore, when a similar adjustment is

Table 3. Influence of lactation type and genotype on selected milk constituents (Means \pm SEM)

Milk component	Lactation type			Genotype		
	Induced	Natural	<i>P</i>	Transgenic	Non-Tg	<i>P</i>
α -Lactalbumin, mg/ml	1.3 \pm 0.1	1.2 \pm 0.1	0.247	1.2 \pm 0.10	1.3 \pm 0.1	0.233
β -Lactoglobulin, mg/ml	6.9 \pm 0.8	4.0 \pm 0.4	0.006	4.6 \pm 0.6	5.7 \pm 0.6	0.084
Lactoferrin, μ g/ml	300.9 \pm 49.4	57.4 \pm 13.6	<0.001	156.5 \pm 35.5	108.9 \pm 33.5	0.320
Lactose, mg/ml	34.0 \pm 2.7	49.3 \pm 1.2	<0.001	40.2 \pm 2.2	49.4 \pm 1.5	0.002
Serum albumin protein, mg/ml†	1.03 \pm 0.26	0.19 \pm 0.20	na‡	1.02 \pm 0.21	0.19 \pm 0.25	na‡
Total protein, mg/ml	45.1 \pm 4.8	20.8 \pm 3.2	<0.001	35.8 \pm 3.5	30.0 \pm 4.5	0.318

† A lactation type \times genotype interaction was detected for serum proteins ($P=0.005$)

‡ na = not applicable

made using β -Ig to normalize lysostaphin concentrations (ovine β -Ig promoter drives the lysostaphin transgene), the results of analysis are also unchanged.

Reproductive performance

A formal evaluation of reproductive performance of the induced females could not be made because the dataset was confounded by cloning. More than half of the animals were either clones or first generation offspring of clones. However, no evidence was observed to suggest the number of services to conception or age at calving differed notably from our herd average.

The induced male (214) and non-induced control bull (303) did not differ in semen parameters such as volume, sperm concentration or motility (results not shown). When sperm from those two bulls was used for *in-vitro* fertilization, no differences were detected in blastocyst production rate (results not shown).

Discussion

Induction success in heifers

Lactation induction methodology has been used for a variety of theoretical and practical reasons including the study of mammary gland development and performance (Narendran et al. 1974; Erb et al. 1976) and as a production tool to stimulate lactation in reproductively incompetent cows (Smith & Schanbacher, 1973; Magliaro et al. 2004). As in this study, lactation induction has been used to assess mammary gland specific transgene performance in cattle (Van Berkel et al. 2002; Brophy et al. 2003), goats (Ebert et al. 1994; Baguisi et al. 1999; Cammuso et al. 2000) and even in pigs (Shamay et al. 1992). The efficiency of what has become the standard oestrogen/progesterone/dexamethasone regime was 89% (8 out of 9 heifers on the first attempt, 100% ultimately) in this study and is similar to the efficiencies of 70–100% reported by others for prepubertal heifers (Smith & Schanbacher, 1973; Fulkerson & McDowell, 1975; Field et al. 1979), sheep (Alifakiotis et al. 1980) and goats

(Cammuso et al. 2000). Furthermore, in the current study there was no evidence that the presence of the transgene influenced the responsiveness of the heifer calves to the induction protocol. It is unclear, from the data presented, whether that was the case in previously published transgenic cattle studies. In the one goat study where influence of the transgene was measured, there was a tendency towards an inverse relationship between level of transgene expression and milk volume collected following induction (Cammuso et al. 2000). Similarly transgenic heifers produced less volume following induction than did the non-transgenic controls. However, in this study a similar inverse dose response relationship was not observed.

Induction success in bull calves

Successful attempts to induce males to lactate have been reported for a 3-month old bull calf (Newstead & Flux, 1973) and for buck goats (Ebert et al. 1994; Cammuso et al. 2000). The bull calf began mammary gland secretions after 50 d of oestrogenic treatment. The success rate of induction of prepubertal (Cammuso et al. 2000) and pubertal (Ebert et al. 1994) transgenic buck goats, using a procedure similar to the one used here, was substantially lower than that achieved for heifer calves or does. Induction success for the post-pubertal bucks (33%) was nearly the same as that observed in this study for our eight prepubertal bull calves (38%). It appears that age is a determining factor in success of induction for both buck goats and bull calves. In this study and a previously published transgenic goat study, only males 6 months of age or older were successfully induced to lactate (Cammuso et al. 2000). Males not responsive to the 2-week standard hormone treatment may be successfully induced by extending the hormone treatment period (Ebert et al. 1994). Computer assisted sperm motion parameters, which have been shown to be positively correlated with *in-vivo* fertility of bull spermatozoa (Farrell et al. 1998; Tardif et al. 1999) were not different for semen from an induced male and his non-induced half sibling. Furthermore, sperm from a hormone-treated bull was fully functional in an *in-vitro* fertilization assay. Admittedly, comparing only two

animals does not offer much assurance that the hormone regime did not have some impact on semen quality, but if it did, it may be of short duration as found with buck goats (Cammuso et al. 2000).

Efficacy of induced lactations as predictor of transgene expression

Transgene product concentration has been successfully detected in induced lactation samples from prepubertal heifers in two studies (Van Berkel et al. 2002; Brophy et al. 2003). Transgene product concentration was said to be virtually identical in induced and natural lactation samples, though quantitative comparisons were not presented. Similar induction studies in transgenic goats (Ebert et al. 1994; Baguisi et al. 1999; Cammuso et al. 2000) yielded varying results, but generally found that induced values were similar to post-parturition levels. In these previous studies, differences in induced and post-parturition levels in does were not detected. However, there was a tendency, in two goat studies, for transgene product concentrations in milk to be higher (numerically, but not statistically) than that in hormonally induced samples (Ebert et al. 1994; Cammuso et al. 2000). We found the opposite to be the case when lysostaphin was measured by ELISA, with the exception of one cow (204) in which lysostaphin concentration did not differ between lactation types.

Though statistical differences in lysostaphin concentration were not detected in samples collected on different days of the induced lactation, concentrations may have diminished if the milking had been extended (Baguisi et al. 1999) and thus more closely match post-parturition lactation values. However, others did not find that to be the case (Ebert et al. 1994). Given the elevated levels of endogenous milk proteins in the induced lactation, it does not seem unreasonable to expect lysostaphin concentrations also to be elevated.

ELISA results described only a modest linear relationship between the concentration of lysostaphin in induced secretions and its concentration in post-parturition milk. However, a moderately strong linear correlation between the lytic activity of the two types of samples was revealed by the SOL assay. Thus, for this transgene product, a functional assay of early induced secretions can serve as a predictor of a cow's production potential during a *post-partum* lactation.

Our ability to predict transgene expression in daughters from induced lactation samples collected from bull calves was thwarted by the low volumes collected from the three responding transgenic bulls. The one bull that yielded adequate volume for replicate ELISA assays had lysostaphin concentrations indistinguishable from that found in an induced heifer of the same line. The concentration in those induced samples was approximately three-times the concentration of lysostaphin in her post-parturition milk. Our ELISA results agree with those of Ebert and colleagues

who found transgene product concentration in induced lactation mammary exudates from bucks to be about twice as concentrated as normally lactating does for the same lines (Ebert et al. 1994). Unfortunately, we did not collect an adequate sample volume to assess the lytic activity of the bull secretions.

Endogenous milk constituents

As in this study, a number of investigators found total protein concentration in samples from induced lactations to be higher than in post-parturition milk (Narendran et al. 1974; Davis et al. 1983; Ball et al. 2000; Magliaro et al. 2004) but others found protein concentration to be similar in samples from the two types of lactation (Cammuso et al. 2000). Ball et al. (2000) found that protein concentration was higher in induced v. natural lactations for heifers that produced small volumes in response to induction. Otherwise, protein concentration was similar between induced and post-parturition lactations. It seems clear that over time the milk constituents of induced lactations approximate postpartum milk (Ball et al. 2000; Kensinger & Magliaro, 2002). The increased total protein seen here and in other studies may be accounted for, in part, by an accumulation of serum proteins in the induced secretion, at least in the early stages of lactation. Several investigators noted the relative abundance of immunoglobulins in induced lactation samples (Newstead & Flux, 1973; Ebert et al. 1994). Our results would also suggest that whey proteins might also contribute to the elevated protein concentrations in induced lactation samples (Table 3). It is interesting to note that while β -lg and LF were clearly elevated in induced lactation samples in this study, α -la was not. That might suggest some differential regulations of those proteins. However, there could be other explanations. Until lactation is established, cell junctions are leaky. This allows albumin into milk. However, the influx/efflux is also related to the size of the protein. Larger proteins do not get out as readily. Since α -la is relatively small, it may be leaking out at a rate that counterbalances the 'concentrating' effects seen at this time with other proteins.

Further, as expected, the presence of the transgene did not alter endogenous milk component composition, except in one unexplained case. Lactose concentrations were significantly less in transgenic animals than in their non-transgenic controls (Table 3). That was the case for both induced secretions and normal milk. Lactose was reduced by approximately 20%. No obvious explanation for how lysostaphin could cause this comes to mind. However, taken together with the concomitant elevated albumin levels in the transgenics suggests that mammary epithelial tight junctions may be leaky. Or, possibly more likely, the low lactose concentration in the transgenics might be a reflection of their low overall *post-partum* milk production.

The present results provide a direct quantitative comparison of induced and post-parturition transgene

expression in the milk from the same animals, demonstrating that contents of secretions from induced lactations are adequate to rank transgenic lines with regard to transgene expression levels during future normal lactations. Furthermore, the data presented here suggest that endogenous milk constituents in induced lactation secretions differ in their relative amounts when compared with the composition of mature post-parturition milk.

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References

- Alifakiotis TA, Katanos I, Hatjiminaoglou I, Zervas N & Zerfiridis G 1980 Induced lactation in dairy ewes by various brief hormone treatments. *Journal of Dairy Science* **63** 750–755
- Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempe MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW & Echeland Y 1999 Production of goats by somatic cell nuclear transfer. *Nature Biotechnology* **17** 456–461
- Baldassarre H, Wang B, Pierson J, Neveu N, Sneek L, Lapointe J, Cote F, Kafidi N, Keefer CL, Lazaris A & Karatzas CN 2004 Prepubertal propagation of transgenic cloned goats by laparoscopic ovum pick-up and in vitro embryo production. *Cloning Stem Cells* **6** 25–29
- Ball S, Polson K, Emeny J, Eyestone W & Akers RM 2000 Induced lactation in prepubertal Holstein heifers. *Journal of Dairy Science* **83** 2459–2463
- Bondioli KR & Wall RJ 1998 Transgenic Livestock. In *Agricultural Biotechnology*, pp. 453–472 (Ed. A Altman). New York: Marcel Dekker, Inc.
- Brophy B, Smolenski G, Wheeler T, Wells DN, L'Huillier P & Laible G 2003 Cloned transgenic cattle produce milk with higher levels of beta-casein and kappa-casein. *Nature Biotechnology* **21** 157–162
- Cammuso C, Porter C, Nims S, Gaucher D, Melican D, Bombard S, Hawkins N, O'Coin A, Ricci C, Brayman C, Buzzell N, Ziomek C & Gavin W 2000 Hormonal induced lactation in transgenic goats. *Animal Biotechnology* **11** 1–17
- Collier RJ, Bauman DE & Hays RL 1976 Effect of reserpine on milk production and serum prolactin of cows hormonally induced into lactation. *Journal of Dairy Science* **60** 896–901
- Davis SR, Welch RA, Pearce MG & Peterson AJ 1983 Induction of lactation in nonpregnant cows by estradiol-17 beta and progesterone from an intravaginal sponge. *Journal of Dairy Science* **66** 450–457
- Ebert KM, DiTullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, Pellerin LJ, Meade HM, Denman J & Roberts B 1994 Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. *Biotechnology* **12** 699–702
- Erb RE, Malven PV, Monk EL, Mollett TA, Smith KL, Schanbacher FL & Willett LB 1976 Hormone induced lactation in the cow. IV. Relationships between lactational performance and hormone concentrations in blood plasma. *Journal of Dairy Science* **59** 1420–1428
- Farrell PB, Presicce GA, Brockett CC & Foote RH 1998 Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology* **49** 871–879
- Field DE, McDowell GH, Buesnel RF & Jessop TM 1979 Artificial induction of lactation in unmated heifers and in heifers with reproductive abnormalities. *Australian Journal of Experimental Agricultural Animal Husbandry* **19** 13–18
- Fulkerson WJ & McDowell GH 1975 Artificial induction of lactation in cattle by use of dexamethasone trimethylacetate. *Australian Journal of Biological Science* **28** 183–187
- Hawk HW & Wall RJ 1994 Improved yields of bovine blastocysts from in vitro-produced oocytes. I. Selection of oocytes and zygotes. *Theriogenology* **41** 1571–1583
- Hirabayashi M, Kodaira K, Takahashi R, Sagara J, Suzuki T & Ueda M 1996 Transgene expression in mammary glands of newborn rats. *Molecular Reproduction and Development* **43** 145–149
- Holt C, Holt WV & Moore HD 1996 Choice of operating conditions to minimize sperm subpopulation sampling bias in the assessment of boar semen by computer-assisted semen analysis. *Journal of Andrology* **17** 587–596
- Kensinger RS & Magliaro AL 2002 Lactation induction. In *Encyclopedia of Dairy Science*, pp. 1447–1452 (Eds H Roginski, JW Fuquary & PF Fox). New York: Academic Press
- Kerr DE, Liang F, Bondioli KR, Zhao H, Kreibich G, Wall RJ & Sun TT 1998 The bladder as a bioreactor: urothelium production and secretion of growth hormone into urine. *Nature Biotechnology* **16** 75–79
- Kerr DE, Plaut K, Bramley AJ, Williamson CM, Lax AJ, Moore K, Wells KD & Wall RJ 2001 Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. *Nature Biotechnology* **19** 66–70
- Logan JS & Martin MJ 1994 Transgenic swine as recombinant production system for human hemoglobin. *Methods in Enzymology* **231** 435–445
- Magliaro AL, Kensinger RS, Ford SA, O'Connor ML, Muller LD & Graboski R 2004 Induced lactation in nonpregnant cows: profitability and response to bovine somatotropin. *Journal of Dairy Science* **87** 3290–3297
- Narendran R, Hacker RR, Batra TR & Burnside EB 1974 Hormonal induction of lactation in the bovine: mammary gland histology and milk composition. *Journal of Dairy Science* **57** 1334–1340
- Newstead DF & Flux DS 1973 Induction of lactation in a bull calf for casein variant phenotyping. *Journal of Dairy Science* **57** 344–346
- Powell AM, Talbot NC, Wells KD, Kerr DE, Pursel VG & Wall RJ 2004 Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biology of Reproduction* **71** 210–216
- Shamay A, Pursel VG, Wall RJ & Hennighausen L 1992 Induction of lactogenesis in transgenic virgin pigs: evidence for gene and integration site-specific hormonal regulation. *Molecular Endocrinology* **6** 191–197
- Smith KL & Schanbacher FL 1973 Hormone induced lactation in the bovine. I. Lactational performance following injections of 17-estradiol and progesterone. *Journal of Dairy Science* **56** 738–743
- Tardif S, Laforest JP, Cormier N & Bailey JL 1999 The importance of porcine sperm parameters on fertility in vivo. *Theriogenology* **52** 447–459
- Van Berkel PH, Welling MM, Geerts M, Van Veen HA, Ravensbergen B, Salaheddine M, Pauwels EK, Pieper F, Nuijens JH & Nibbering PH 2002 Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nature Biotechnology* **20** 484–487
- Wall RJ, Powell AM, Paape MJ, Kerr DE, Bannerman DD, Pursel VG, Wells KD, Talbot N & Hawk HW 2005 Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nature Biotechnology* **23** 445–451